ANTIGENIC PROPERTIES OF TYPE E CL. BOTULINUM PROTOXIN

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It has been established by the investigations of a number of authors (Duff and coauthors, 1956; Gordon and coauthors, 1957; Matveyev, 1959) that Cl. botulinum type E, as well as Cl. perfringens type D and E (Turner and Rodwell, 1943; Ross and coauthors, 1949), during incubation on liquid nutrient media formed a toxin and a protoxin. The latter may be activated by various enzymes (trypsin, proteinase of Cl. sporogenes, yeast proteinase, etc.) as a result of which the power of the toxin is increased by many times.

It is known that botulinum toxins of all types, including type E, possess high antigenic and immunogenic properties. In the present article materials are presented which testify to the antigenic properties of the protoxin of type E Cl. botulinum protoxin which are equal to the antigenic properties of the toxin.

The work was conducted with twelve strains of Cl. botulinum type E which were incubated on casein fungus medium for 5-7 days at 28°. The minimal lethal dose of the toxins was determined on white mice following intravenous introduction.

For the neutralization reaction we used the standard anti-botulinum serum type E, prepared at the Tarasevich State Control Institute and which contained 100 AE in 1 ml, and also a native horse antitoxic-antibotulinum serum type E, which was prepared by us and which contained 1200 AE in 1 ml.

The filtrates Cl. botulinum type E were activated with a 4% solution of pancreatin, for which we combined two parts of the filtrate

of a type E culture and 1 part of a solution of pancreatin. The mixture was maintained at 37° for 1 hour.

It had been established that 5-7 day filtrates of various strains of Cl. botulinum type E may be activated by pancreatin, and that the biological activity (strength of the toxin) was increased by 10 to 1,000 times (see our previous works). This means that in the non-activated filtrate of Cl. botulinum type E a large part of the specific protein is maintained in a biologically inactive condition in the form of protoxin and only 1/1000th to 1/10th a part of it - in the form of a poisonous substance - the toxin.

The toxins which were obtained after activation were strictly type specific and were neutralized in tests on mice only by the antitoxic antibotulinum serum type E.

In the indirect hemagglutination reaction with activated and non-activated toxins a positive response was obtained in the same dilutions of the filtrates. This testified to the same antigenic activity of activated and non-activated toxins though the biological activity on animals in the first was increased after activation by 10 to 100 times (table 1).

Thus, the toxin E-188-20 prior to activation contained in 1 ml. 1,000 and after activation 100,000 Dlm, but in both cases the reaction was positive with a dilution of the toxin to 1:64,000, that is, the amount of antigen after activation was not increased. This should have been expected since it is impossible to assume that under the action of a proteolytic enzyme there was an increase in the amount of specific antigen in the filtrate, though an increase took place in the toxicity of the filtrate. In several tests, on the contrary, in spite of an increase of toxicity, the indirect hemagglutination reaction with activated filtrates was 1-2 dilutions weaker.

In the following tests when setting up the neutralization reaction on mice with activated and non-activated toxic filtrates, it was noted that for the neutralization of the same volume of native toxin type E (in the test 1 ml. of toxin of the same series was always used), taken before and after its activation by pancreatin, it required approximately the same (or all told 1½ to 2½ times more) amount of antitoxic units (reaction at room temperature), at the same time that the lethal activity of the toxin increased after activation in these tests by 10 to 100 times.

When setting up the test under the conditions of room temperature (table 2) for the neutralization of 1 ml. of non-activated toxin, which contained 50 Dlm/ml, two AE of serum was necessary, and for the neutralization of 1 ml. of activated toxin, containing 1,000 Dlm (index of activation 20), 3 AE were necessary. In other words, in non-activated toxin 1 AE neutralized 50 Dlm, and in activated - 330 Dlm.

When setting up the reaction of antitoxin binding on white mice with non-activated and activated filtrates, we observed the same regularity (see table 2). If with the non-activated filtrate the reaction of antitoxin binding was positive in a dose of 0.05 - 0.03 ml (which corresponded to 2-3 EC in 1 ml.), then with activated, a positive response was obtained in a dose of 0.03 - 0.02 ml. (3-5 EC in 1 ml), though the toxicity of the latter was greater by 10 to 100 times. This insignificant increase of antigenic properties of the toxin after activation is not true and was caused by the continuation of the action of the enzyme under the conditions of room temperature at which the reactions of neutralization and antitoxin binding was set up. If they were conducted at 4°, when the action of the enzyme was cut off, then for the neutralization of both the non-activated as well as the activated toxins, it required the same amount of antitoxic units of serum, and with both toxins similar results were obtained in the antitoxin binding reaction.

Chertkova and coauthors (1960), during the titration of a test dose of various series of the standard botulinum toxins type E, which is equivalent of 0.1 AE of the standard antibotulinum serum, established that the test dose of the toxin contained 5 Dlm, if the toxin was not preliminarily activated, and 60 Dlm if the toxin was activated during the process of its preparation, i.e., in the first case 0.1 AE neutralized 5 Dlm, and in the second - 60 Dlm.

The test results obtained by us, as well as the data from Chertkova, speak for the antigenic, i.e., the antitoxin binding, properties of the protoxin of Cl. botulinum type E. The protoxin possessed antigenic properties which were equivalent to the antigenic properties of the toxin.

These pecularities of the botulinum toxin type E must be taken into consideration when testing the intensity of immunity in actively or passively immunized animals. Ignorance of them may lead to the obtaining of contradictory and even mistaken results. As it is seen

from our tests, the same amount of AE of serum will neutralize significantly more Dlm of activated toxin than of non-activated toxin, and the use of activated toxins exhibits a higher intensity of immunity than the administration of non-activated toxin.

In another series of our tests on the preparation of botulinum anatoxins type E for the immunization of horses and other animals we obtained data which speaks also for the antigenic properties of protoxin (1959). All together 16 series of anatoxin were obtained. The anatoxins were prepared both from non-activated (7 series), as well as from toxins which were activated by pancreatin (3 series) or Cl. sporogenes (6 series). The activated toxins contained 50,000 - 100,000, and the non-activated - 1,000 - 2,000 Dlm in 1 ml. After being rendered completely harmless by Formalin at 38°, the antitoxin binding properties of both antitoxins fluctuated within limits of 3-5-10 AE (on a calculation for 1 AE) regardless of the strength of the toxin, though based on the strength of the toxin, the activated and non-activated toxins differed by 50 to 100 times. All this may be explained by the fact that the protoxin, just as the toxin, following treatment by Formalin and heat, preserved the antigenic properties, therefore, its activation by pancreatin, that is, the transition into a toxin before being rendered harmless, apparently has no significance for increasing the antigenic activity of the type E anatoxin. During the immunization of horses with the aim of obtaining pharmaceutical diagnostic antitoxic, antibotulinum sera type E, we did not note any differences in the titers in the horses, immunized with the anatoxins obtained from activated and non-activated toxins.

Vorobyev and coauthors (1959) established that no dependency exists between the activity of the toxin of Cl. botulinum type E, expressed by the number of Dlm/ml, and its antitoxin binding properties. Thus, 1 EC (EC to 0.1 AE) corresponded with the following number of Dlm of the toxin of Cl. botulinum type E: a) In native and concentrated non-activated pure cultures - 1^{-10} Dlm; b) in native and concentrated mixed cultures - 500 to 1500 Dlm; c) in native and concentrated cultures, activated by pancreatin - 400 to 1000 Dlm. Regardless of the relationship of the Dlm/EC in the native or in the concentrated toxin after its being rendered harmless with Formalin, they obtained anatoxins having similar antitoxin binding properties.

In this manner the data presented of our own investigations and the data of other authors testify to the presence in the protoxin of Cl. botulinum type E of antigenic properties equivalent to the antigenic properties of the toxin.

In the light of these facts, the results of Khatuntsev's tests (1960) with botulinum toxin type E become fully understandable.

In these tests the author, while studying the summation of toxic stimulation during the daily introduction to white mice of 1/10th Dlm, observed the death of approximately 30% of the animals and the development of immunity in the remainder (the animals endured 2-5 Dlm of type E toxin).

All this may be explained by the fact that the type E toxin always contains a significant amount of protoxin, which during the repeated administration also causes immunity in animals considerably more rapidly than is observed in tests with toxins (botulinum types A and C), which do not contain protoxin in the 5-8 day filtrate.

Conclusions

1. In the tests with twelve strains of Cl. botulinum type E it was established that in liquid nutrient media this microbe produces a toxin and a protoxin. The latter converts into a toxin under the action of several enzymes. The titer of the toxin following activation was considerably increased, and the index of activation fluctuated within the limits of 10 up to 1,000.

2. For the neutralisation of the same volumes of native toxin of type E before and after activation by enzymes the same amount of antitoxic units was required, while the lethal activity of the toxin following activation increased by 10 to 100 times, i.e., the antigenic properties of the protoxin were equivalent to the antigenic properties of the toxin.

3. With the type E botulinum toxin before and after activation an indirect hemagglutination reaction was obtained in the same dilutions, in spite of the fact that the activity of the toxin following activation was increased by many times, which also testified to the equivalence of the protoxin and the toxin.

4. The data obtained when studying the antitoxin binding capability of the toxins and anatoxins of type E also testified to the presence of antigenic properties in the protoxins of type E.

The anatoxins obtained from botulinum toxins type E both of non-activated as well as of toxins activated by pancreatin or Cl. sporogenes, possessed the same antitoxin binding properties when the reaction was set up on white mice.

Literature

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Table 1

Indirect hemagglutination reaction with type E botulinum toxin before and after activation with pancreatin

Serum	No. strain	Time of setting up the reaction	Amount of Dlm in 1 ml	Result of the reaction with various dilutions of toxin									Control of serum
				1:1 000	1:2 000	1:4 000	1:8 000	1:16 000	1:32 000	1:64 000	1:128 000	1:256 000	
Type E series No 162	188-20	Before activation	1000	+++	+++	+++	+++	+++	+++	+++	++	+	-
		After activation	100000	+++	+++	+++	+++	+++	+++	+++	+	±	-
	Ped-20	Before activation	10	+++	+++	+++	+++	+++	+++	+++	+	-	-
		After activation	1000	+++	+++	+++	+++	+++	++	+	+	-	-
	115-35	Before activation	100	+++	+++	+++	+++	+++	+++	++	++	±	-
		After activation	1000	+++	+++	+++	+++	+++	+++	+++	+	-	-

Legend: +++, ++, + positive reaction of various intensities, ± doubtful, - negative reaction

Table 2

Results of the neutralization and antitoxin-binding reactions of nonactivated and activated type E botulinum toxin

Time the reaction was set up	Neutralization reaction					Antitoxin-binding reaction			Control
	Volume of nonactivated filtrate (in ml)	Amount of pancreatin or physiological solution (in ml)	Amount of serum (in AE)	Results at 20°	Results at 4°	Dose of filtrate (in ml)	Results at 20°	Results at 4°	Titer of toxin (in Dlm)
Before activation	1	Physiological solution 0.5	4	00	00	0.05	++	++	20-++
	1	0.5	3	00	00	0.03	++	++	50-++
	1	0.5	2	00	00	0.02	00	00	100-00
	1	0.5	1	++	++	0.01	00	00	200-00
After activation	1	Pancreatin 0.5	4	00	00	0.05	++	++	500-++
	1	0.5	3	00	00	0.03	++	++	1000-++
	1	0.5	2	++	00	0.02	++	00	2000-00
	1	0.5	1	++	++	0.01	00	00	5000-00

Legend: ++ mice died, 00 mice healthy.
AE = antigen units.